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Role of MAP Kinases in the 1,25-Dihydroxyvitamin D_3-induced Transactivation of the Rat Cytochrome P450C24 (CYP24) Promoter

SPECIFIC FUNCTIONS FOR ERK1/ERK2 AND ERK5*

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The current study investigated the action of 1,25-dihydroxyvitamin D_3 (1,25D) at the genomic and signal transduction levels to induce rat cytochrome P450C24 (*CYP24*) gene expression. A rat *CYP24* promoter containing two vitamin D response elements and an Ets-1 binding site was used to characterize the mechanism of actions for the 1,25D secosteroid hormone. The Ets-1 binding site was determined to function cooperatively with the most proximal vitamin D response element in a hormone-dependent fashion. Evidence was obtained for distinct roles of ERK1/ERK2 and ERK5 in the 1,25D-inductive actions. Specifically, 1,25D stimulated the activities of ERK1/ERK2 and ERK5 in a Ras-dependent manner. Promoter induction was inhibited by mitogen-activated protein (MAP) kinase inhibitors (PD98059 and U0126) and a dominant-negative Ras mutant (Ras17N). Induction of *CYP24* by 1,25D was also inhibited by overexpression of dominant-negative mutants of ERK1 and MEK5 (ERK1K71R and MEK5(A)). The p38 and JNK MAP kinases were not required for the action of 1,25D. 9-*cis* retinoid X receptor α (RXRα) interacted with ERK2 but not ERK5 in intact cells, whereas Ets-1 interacted preferentially with ERK5. Increased phosphorylation of RXRα and Ets-1 was detected in response to 1,25D. Activated ERK2 and ERK5 specifically phosphorylated RXRα and Ets-1, respectively. Mutagenesis of Ets-1 (T38A) reduced *CYP24* promoter activity to levels observed with the dominant-negative MEK5(A) and inhibited ERK5-directed phosphorylation. Mutated RXRα (S260A) inhibited 1,25D-induced *CYP24* promoter activity and abolished phosphorylation by activated ERK2. The 1,25D-inductive action through ERK5 involved Ets-1 phosphorylation at threonine 38, whereas hormone stimulation of ERK1/ERK2 required RXRα phosphorylation on serine 260. The ERK1/ERK2 and ERK5 modules provide a novel mechanism for linking the rapid signal transduction and slower transcription actions of 1,25D to induce *CYP24* gene expression.

The hormonally active form of vitamin D_3 is 1,25-dihydroxyvitamin D_3 (1,25D).[1] This secosteroid hormone plays a central role in calcium homeostasis and bone metabolism and participates in a diverse range of cellular actions including inhibition of tumor cell growth, induction of cell differentiation, and modulation of the immune response (1–4). The transcriptional actions of 1,25D are mediated by the nuclear vitamin D receptor (VDR), which heterodimerizes with retinoid X receptor (RXR) and binds to specific vitamin D response element (VDRE) sites in the promoter region of vitamin D-responsive genes (5). Transactivation by the liganded VDR/RXR is dependent upon the binding of one or more coactivator complexes that permit bridging to the RNA polymerase II machinery (6, 7). In the unliganded state, some nuclear receptors including VDR (8) can bind a co-repressor that inhibits transactivation (9, 10), but this repressor dissociates upon ligand binding.

Maintenance of cellular 1,25D levels is critical to regulation of the function of the hormone in which high levels of 1,25D are toxic and can lead to hypercalcemia and systems failure. Therefore, a highly specific hydroxylase system functions within the vitamin D pathway to tightly regulate control of 1,25D levels. The mitochondrial hydroxylases cytochromes P450C1 and P450C24 are responsible for the bioactivation and degradation of vitamin D_3 metabolites, respectively (11–13). Genes for the hydroxylase enzymes are subject to stringent hormonal control, most notably by 1,25D, which functions to down-regulate P450C1 and up-regulate P450C24 enzyme expressions.

We are studying the molecular regulation of the *P450C24* (*CYP24*) gene, which is expressed widely in different cell types with the highest activity observed in kidney (1). CYP24 is a multifunctional enzyme that directs the side-chain oxidation and cleavage of 1,25D and 25D to catabolic carboxylic acid end products (11, 13). The action of 1,25D to induce expression of the rat *CYP24* gene is mediated by two VDREs that are located

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[1] The abbreviations used are: 1,25D, 1,25-dihydroxyvitamin D_3; 25D, hydroxyvitamin D_3; VDR, vitamin D receptor; VDRE, vitamin D response element; EBS, Ets-1 binding site; RXR, 9-*cis* retinoid X receptor; P450C1, 25-hydroxyvitamin D-1-hydroxylase; P450C24, 25-hydroxyvitamin D-24-hydroxylase; CYP24, cytochrome P450C24; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK kinase; MEKK, MEK kinase; JNK, c-Jun NH_2-terminal kinase; MKP-1, MAP kinase phosphatase-1; MBP, myelin basic protein; HA, hemagglutinin; Luc, luciferase; GST, glutathione *S*-transferase; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*,-trimethylammonium methylsulfate; TK, thymidine kinase; RSV, Rous sarcoma virus; RL, *Renilla* luciferase; TK, thymidine kinase; h, human; .

about 100 bp apart within the −300-bp promoter region. The synergistic action of the tandemly arranged VDREs underlies the extraordinary high levels of induction that can be attained in response to 1,25D and represents the most active vitamin D-dependent promoter identified to date (14–16). The important role for *P450C24* gene expression has been further demonstrated in *CYP24* knockout mice in which null animals demonstrated high ambient 1,25D levels and bone mineralization defects (17).

During our studies into the molecular mechanism by which 1,25D induces *CYP24* promoter activity, we identified an Ets-1 binding site located near the proximal VDRE that is important for maximal transcriptional induction. Exogenously expressed Ets-1 stimulated vitamin D induction, and this was further enhanced by coexpression of Ras (18). Ras can elicit activation of several downstream mitogen-activated protein (MAP) kinases, among other signaling molecules (19–21), and in the present study, we have asked whether vitamin D increases the activities of MAP kinase pathways and whether these pathways impinge on the 1,25D-induced activation of the *CYP24* promoter. Recent studies have indicated that 1,25D can elicit rapid responses, apparently independent of gene expression (22, 23). These non-genomic effects include changes in ceramide/phosphoinositide metabolism and increases in intracellular calcium as well as activation of protein kinase C (24). Presumably these vitamin D-dependent responses are mediated by a vitamin D receptor at the plasma membrane (25), but the relationship of this putative receptor with that in the nucleus remains obscure.

We provide evidence in the current study that vitamin D directs the rapid activation of the MAP kinases ERK1/ERK2 and ERK5. Activation of RXRα was obligatory to ERK1/ERK2, whereas ERK5 was specifically required for Ets-1 phosphorylation. Inhibition of either the ERK1/ERK2 or ERK5 modules substantially reduced vitamin D-induced transcriptional activation of the *CYP24* promoter. Furthermore, simultaneous inhibition of both of these modules abrogated *CYP24* promoter activity induced by 1,25D, an effect that was mimicked by inhibition of Ras function. Our results emphasize the critical roles played by MAP kinase pathways in the phosphorylation of Ets-1 and RXR during the 1,25D induction process.

EXPERIMENTAL PROCEDURES

Reagents—1,25D and 25D were purchased from Tetrionics, Inc., Madison, WI. The Dual-Luciferase Reporter Assay System (DLR) and the anti-ACTIVE™ ERK antibody were from Promega (Madison, WI), and anti-HA and anti-ERK2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-phosphothreonine and anti-phosphoserine antibodies were purchased from Sigma. [γ-^{32}P]ATP (specific activity, 4000 Ci/mmol) was obtained from Geneworks (Adelaide, Australia). Myelin basic protein (MBP) was purchased from Sigma. DOTAP was obtained from Roche Diagnostics. The following inhibitors, SB203580, PD98059, and U0126, were purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

Promoter Luciferase Constructs and Expression Plasmids—CYP24 promoter luciferase constructs used in this study contain −298 bp of CYP24 promoter sequence together with 74 bp of 5′-untranslated region in the pGL3 basic vector with the firefly luciferase reporter gene as described previously (see Fig. 1) (18). The mutant constructs, pCYPmVDRE1(−298)-Luc, pCYPmVDRE2(−298)-Luc, and pCYPmVDRE1 + 2(−298)-Luc, have been described previously (18). VP16-VDR, pG5E1B-Luc, pRSV-hVDR, and pRSV-hRXRα constructs have been described previously (9, 16, 18, 26). GAL4-Ets-1 was constructed by PCR amplification of human Ets-1 cDNA sequence using pEFBOS-Ets-1 as a template (18) and two PCR primers engineered with an *Nde*I restriction enzyme site. The PCR product was cloned at the *Nde*I site in the GAL4 vector (9). GAL4-Ets-1T38A and pRSV-hRXRα S260A were created in our laboratory by the QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). The dominant-negative Ras (Ras17N) and dominant-negative ERK1 (ERK1K71R) have been described (27, 28). The dominant-negative MEK5, MEK5(A), and HA-ERK5 constructs were kindly provided by Dr E. Nishida (Kyoto University, Kyoto, Japan) (29). GST-Ets-1 was provided by Professor Ismail Kola, and the GST-Ets-1T38A mutant was constructed in our laboratory. Expression vectors for GST-hRXRα and GST-hVDR have been described previously (18). The GST-tagged proteins were purified using glutathione-Sepharose 4B (Qiagen, GmbH, Hilden, Germany). The MEKK-1 dominant-negative and HA-ERK2 clones were kindly provided by Dr. David Riches (Denver, CO) (30), and the MAP kinase phosphatase-1 (MKP-1) overexpression clone has been described previously (31).

Maintenance and Transfection of Cells—Monkey kidney fibroblast COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. In preparation for transfection, cells were grown in a 75-cm^2 flask to 60–70% confluency, washed once with phosphate-buffered saline, and removed by trypsinization. Cells (40,000/well) were seeded in a 24-well tray and grown in 400 μl of DMEM supplemented with 5% fetal calf serum. After the cells were attached in the well (3–4 h), the medium was removed, and the cells were washed once with phosphate-buffered saline, and then the medium was replaced with serum-free RPMI medium. Cells were incubated for 2 h for synchronization prior to transfection. For each triplicate transfection, the CYP24 promoter luciferase construct (600 ng) was mixed with 15 μl of Hepes buffer (20 mM, pH 7.4) in an Eppendorf tube with 600 ng of Ets-1 expression clone (pEFBos-Ets-1, Ref. 18) or vector alone or the indicated amount of dominant-negative expression clones together with 150 ng of pRL-TK as a control for transfection efficiency. For most experiments, 600 ng of pRSV-hVDR was also transfected to compensate for the low endogenous VDR level. In all transfections, the final amount of DNA was kept constant by addition of the appropriate vector plasmid. In another tube, 7.5 μg of DOTAP was diluted to 15 μl in Hepes buffer and mixed with 15 μl of diluted DNA plasmids. After incubation for 10 min at room temperature for DNA·DOTAP complex formation, 10 μl of this DNA·DOTAP complex was aliquoted into three wells of a 24-well plate. The following day, the medium was replaced with 400 μl of serum-free RPMI medium, and cells were further cultured for 24 h in the presence of a specific kinase inhibitor and/or 1,25D or ethanol carrier prior to harvesting. Dual luciferase activity in cell lysates was determined by the DLR assay kit using a luminometer model TD 20/20 (Turner Design Instruments, Sunnyvale, CA).

Mammalian Two-hybrid Assay—Human choriocarcinoma cells, JEG-3, were cultured for 24 h in DMEM containing 5% charcoal-stripped fetal calf serum. Cells were cotransfected with 200 ng of reporter plasmid (pG5E1b-Luc) and 50 ng of pRL-TK-Luc (transfection efficiency control) with 500 ng each of GAL4-Ets-1 or GAL4-Ets-1T38A plasmids with either VP16-TR or VP16-VDR expression plasmids using the DOTAP transfection protocol as described earlier in this section. After 24 h, medium was changed, and 1,25D was added as indicated. Cells were further cultured for 24 h in serum-free DMEM and then harvested for DLR assays.

Preparation of Cell Lysates—COS-1 cells with or without transfected HA-tagged expression constructs (confluent 10-cm dishes) were stimulated with 1,25D, H_2O_2, or phorbol 12-myristate 13-acetate for the times indicated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The incubations were terminated by removing the medium and washing the cells once with Hanks' buffered salts solution (4 °C). The cells were scraped and lysed in 200 μl of buffer A (25 mM Tris/HCl, pH 7.5, 2 mM EGTA, 25 mM NaCl, 1 mM Na_3VO_4, 38 mM *p*-nitrophenyl phosphate, 10 μg/ml each of pepstatin A, benzamidine, aprotinin, and leupeptin, 2 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol) by constant rotation for 2 h at 4 °C. Soluble fractions were collected after centrifugation (16,000 × *g*, 3 min, 4 °C), and the protein content of the lysates was determined by Lowry's method of protein determination. Samples were stored at −70 °C until assayed. For Western blot analysis, samples were mixed with Laemmli buffer and boiled before being stored.

Immunoprecipitation—Lysates containing equal amounts of protein were precleared with protein A-Sepharose (15 μl) before being incubated with anti-HA antibody (3 μg of each) to immunoprecipitate HA-ERK2, HA-ERK5, HA-RXRα, or HA-Ets-1. The antigen-antibody complexes were sedimented by the addition of protein A-Sepharose (15 μl) and centrifugation (16,000 × *g* for 20 s, 4 °C). The immune complexes were washed (2 × 200 ml) with buffer A.

Kinase Assays—The immune complexes were washed with assay buffer (1 × 200 μl) (20 mM HEPES, pH 7.2, 20 mM glycerophosphate, 10 mM Sigma 104, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM orthovanadate, 20 mM cold ATP), and kinase assays were started by the addition of assay buffer (30 ml) containing 10 μCi of [γ-^{32}P]ATP and MBP, GST-RXR, GST-Ets-1, His-Ets-1, or GST-VDR (10–15 μg/sample). In

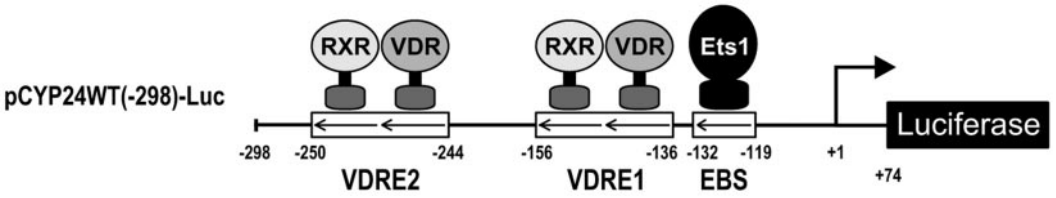

FIG. 1. **Schematic of wild type pCYP24WT(−298)-luciferase reporter construct.** Relative locations of the VDRE-1 and VDRE-2 and EBS located on the antisense strand of the rat cytochrome P450C24 (*CYP24*) promoter are shown. The construct contains −298 bp of promoter sequence together with 74 bp of 5′ untranslated region.

some assays, GST-tagged mutants of RXR or Ets-1 were used. After 20 min at 30 °C, the assays were terminated by the addition of Laemmli buffer (15 ml), and the samples were boiled for 5 min. Phosphorylated substrates were fractionated on 16% (MBP) or 10% (GST-tagged proteins) SDS-PAGE gels and detected using an Instant Imager (Packard Instruments, Canberra, Australia).

Western Blot Analysis—To determine the level of ERK1 and ERK2 dual phosphorylation, lysates containing equal amounts of denatured proteins were subjected to SDS-PAGE (12% gels). After transfer to nitrocellulose, the blot was probed with anti-ACTIVE™ ERK antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (32). The blot was stripped using a Western blot recycling kit (Alpha Diagnostic International, San Antonio, Texas) and reprobed with anti-ERK2 antibody to confirm equal protein loading between lanes. To detect co-immunoprecipitated proteins, immunoprecipitated samples were subjected to Western blot analysis, and the blots were probed first with one antibody, stripped, and reprobed with another antibody as indicated under "Results."

RESULTS

Stimulatory Action of Ets-1—The plasmid pCYP24WT(−298)-Luc contains −298 bp of rat *CYP24* promoter sequence encompassing the two known functional VDREs and the Ets-1 protein binding site designated EBS (Fig. 1). We have previously shown that Ets-1 present in nuclear extracts of COS-1 cells binds EBS, whereas exogenous Ets-1 can stimulate 1,25D induction of a truncated *CYP24* promoter containing only the first VDRE and the nearby EBS (18). Initial studies were undertaken to evaluate the effect of ectopically expressed wild type Ets-1 on 1,25D induction of the −298 bp *CYP24* promoter. With hormone alone, a 4.8-fold level of induction was observed, and overexpressed Ets-1 increased this to 11.2-fold (Fig. 2*A*). Endogenous VDR is limiting in these cells, and exogenously expressed human VDR (hVDR) increased 1,25D induction to 26-fold, whereas a combination of exogenous VDR and Ets-1 further enhanced induction to 56-fold, the latter reflecting a functional cooperation of these transcription factors. When the EBS was inactivated (pCYP24mEBS(−298)-Luc), the level of induction by exogenous Ets-1 and VDR together was reduced to 14.0-fold (Fig. 2*A*). Ets-1 alone did not alter basal promoter activity in the absence of hormone (data not shown).

A mutant form of the wild type Ets-1 protein was examined in which threonine residue 38 was mutated to alanine (Ets-1 T38A). This threonine residue is conserved in several Ets proteins, and based on other work (31), it is likely to be phosphorylated in a Ras-dependent fashion, although the identity of any specific MAP kinase involved is not known (33). The 26-fold level of induction observed with exogenous VDR (Fig. 2*A*) was lowered to 16-fold when the mutant Ets-1 T38A was overexpressed, a value comparable with that seen when the EBS was mutated (Fig. 2*A*). This result shows that the expressed mutant Ets-1 protein competes strongly with endogenous Ets proteins for binding to EBS but is unable to functionally cooperate with VDR·RXR complexes on either VDRE-1 or VDRE-2 in response to 1,25D. In control experiments, it was established that Ets-1 T38A did not alter basal expression of the construct (results not shown), whereas mutagenesis of both VDREs abolished 1,25D induction (Fig. 2*A*). These data demonstrate that wild type Ets-1 substantially enhance vitamin D-induced promoter activity in the presence of two VDREs and that the threonine 38 residue of Ets-1 is vital for its functional cooperation with VDR in the presence of 1,25D.

Experiments were undertaken to determine whether the functional cooperation observed between exogenously expressed hVDR and Ets-1 occurred on one or both VDREs present in the *CYP24* promoter. As observed already, overexpression of hVDR alone gave a 26-fold level of induction with pCYP24WT(−298)-Luc, and this was increased to 56-fold with coexpression of Ets-1 (Fig. 2*B*). There was no stimulatory effect of overexpressing Ets-1 when VDRE-1 was mutated (Fig. 2*B*); however, over-coexpression of Ets-1 and hVDR increased 1,25D induction from 7.9- to 14.9-fold when analyzed in the presence of inactive VDRE-2 (Fig. 2*B*). The results demonstrate that the proximity of the EBS to VDRE-1 underlies its contribution to induction. Evidence for an *in vivo* protein-protein interaction between Ets-1 and VDR was achieved using the mammalian two-hybrid assay and the plasmid G5E1B-Luc as reporter (Fig. 2*C*). The fusion protein GAL4-Ets-1 interacted substantially with VP16-VDR but did not interact with control VP16-T3R (Fig. 2*C*). A similar interaction was seen with GAL4-Ets-1T38A (results not shown). VDR and wild type and mutant Ets-1 interactions were unaltered by 1,25D treatment (Fig. 2*C*). It is evident from these experiments that the ligand-independent interaction between Ets-1 and VDR does not require the threonine 38 residue of Ets-1. In agreement with this observation, it has been shown from *in vitro* pull-down experiments that a region in the DNA binding domain of VDR interacts with an Ets-1 deletion protein that lacks threonine 38 (34).

Suppression by Dominant-negative Ras and EBS Mutations—We have previously shown that overexpression of H-Ras in COS-1 cells increases 1,25D induction of the *CYP24* promoter construct and that this action is potentiated in the presence of the wild type but not the mutant form Ets-1 (18). This implies that Ras plays a role in the 1,25D induction of the *CYP24* promoter in COS-1 cells and that Ets-1 can positively modulate the action of Ras. To investigate the possible involvement of endogenous Ras activity, we have overexpressed an inhibitory dominant-negative *ras* construct, *ras*17N. Expression of Ras17N strongly reduced 1,25D induction of the pCYP24WT(−298)-Luc construct from 26- to 3.5-fold at the highest concentration tested. At these concentrations of Ras17N, there was no effect on basal expression of pCYP24WT(−298)-Luc or expression of the internal control pRL-TK-Luc described under "Experimental Procedures" (data not shown), demonstrating that Ras17N has a selective inhibitory effect on 1,25D induction. However, in the absence of Ras17N, mutagenesis of the EBS reduced 1,25D induction to 14.0-fold (Fig. 3). These results not only demonstrate that Ras is essential for components of the 1,25D-responsive transcription machinery but also suggest that although the EBS is a downstream mediator site of Ras signaling, the action of Ras is also channeled through other downstream effectors.

Activation of ERK1/ERK2 and ERK5 by 1,25D—The extracellular signal-regulated protein kinase ERK1/ERK2 MAP

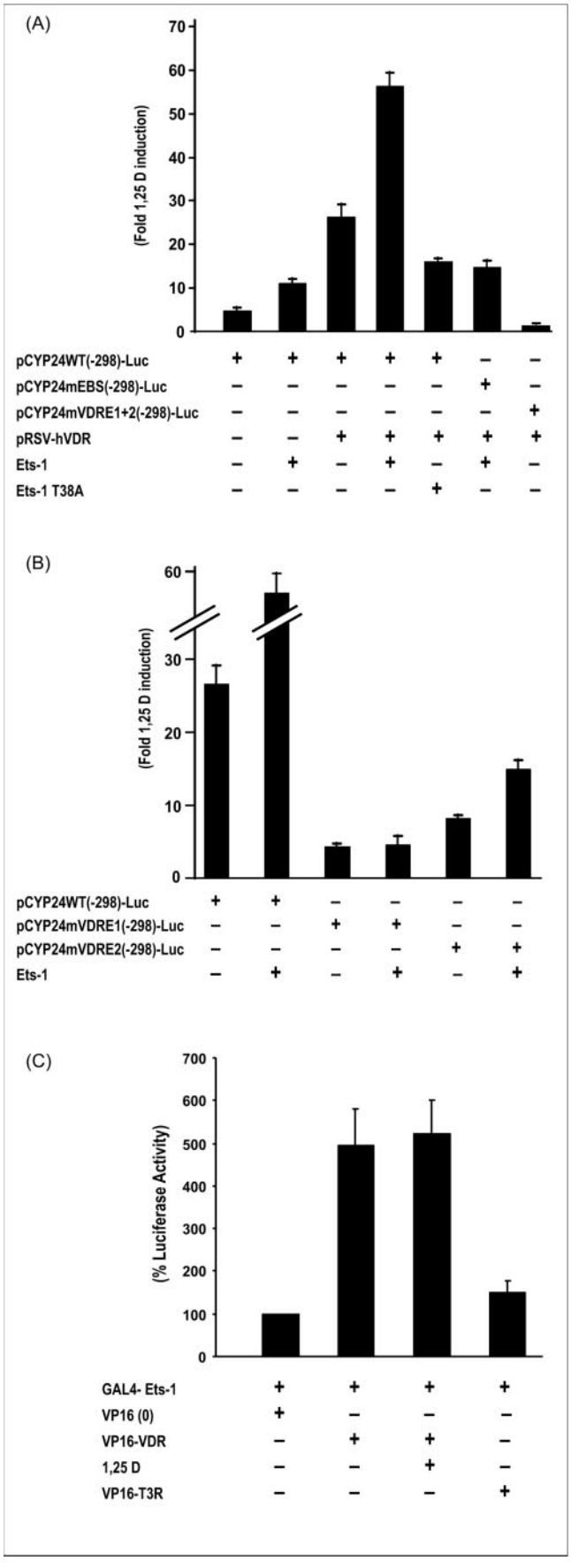

FIG. 2. **Role of Ets-1 in CYP24 promoter function.** *A*, effect of Ets-1 on 1,25D-dependent transactivation of *CYP24* promoter-luciferase constructs. pCYP24WT(−298)-Luc, pCYP24mEBS(−298)-Luc, or pCYP24mVDRE1 + 2(−298)-Luc constructs were cotransfected into COS-1 cells together with expression clones for Ets-1 and Ets-1T38A as indicated. To compensate for low endogenous VDR, human VDR (*pRSV-hVDR*) was cotransfected in all experiments. As an internal control for correction of transfection efficiency, cells were also transfected with pRL-TK-Luc (see "Experimental Procedures"). Each construct was tested for 1,25D (10^{-7} M) transactivation, and the level of induction (*Fold 1,25D induction*) is shown as the ratio of luciferase activity from 1,25D-treated cells to that of untreated cells. The data presented here are the averages of three independent experiments ± S.D. *B*, Ets-1 stimulates 1,25D-dependent transactivation of the *CYP24* promoter through VDRE-1. pCYP24WT(−298)-Luc, pCYP24mVDRE-1(−298)-Luc, or pCYP24mVDRE2(−298)-Luc constructs were cotransfected into COS-1 cells together with pRSV-hVDR (not shown). The promoter constructs were tested for 1,25D transactivation (as in *panel A*) in the presence (+) or absence (−) of overexpressed Ets-1. The data presented here are the averages of three independent experiments ± S.D. *C*, protein-protein interaction of Ets-1 with VDR in the mammalian two-hybrid assay. Human choriocarcinoma cells (JEG-3 cells) were cotransfected with 500 ng of GAL4-Ets-1 chimera and 500 ng of VP16 vector (*VP16* (*0*)) or VP16-VDR or VP16-T3R chimera as indicated together with 200 ng of pG5E1B-Luc reporter with 50 ng of pRL-TK-Luc as an internal control. Cells were also treated with 1,25D (10^{-7} M) as indicated. The luciferase activity of GAL4-Ets-1 in the presence of VP16 vector is arbitrarily set to 100%, and activities are expressed relative to this as percent of expression. The data presented here are the averages of three independent experiments ± S.D.

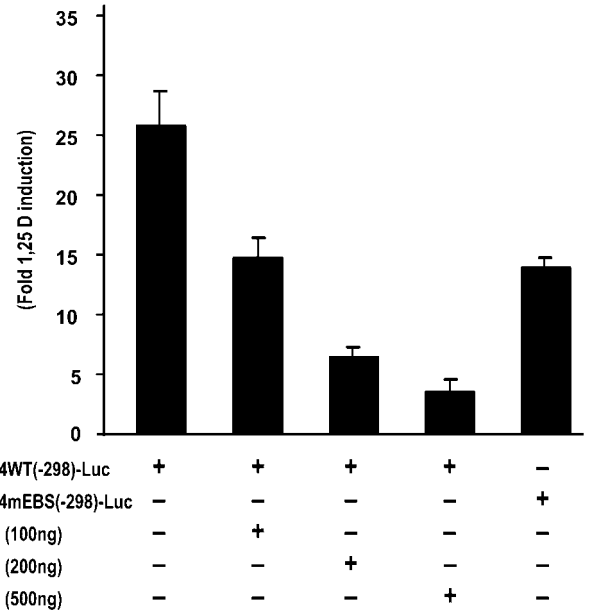

FIG. 3. **Inhibition of 1,25D-dependent transactivation of *CYP24* promoter-luciferase constructs by the *ras* dominant-negative construct, *ras*17N.** pCYP24WT(−298)-Luc, pCYP24-mEBS(−298)-Luc, or pRSVhVDR (not shown) were cotransfected in COS-1 cells together with increasing doses of *ras*17N expression clone (100–500 ng). The promoter constructs were tested for 1,25D transactivation (as in *panel A* of Fig. 2). The data presented here are the averages of three independent experiments ± S.D.

kinases are major downstream effectors of Ras. Both MAP kinases are members of a cascade of kinases that consists of the MAP kinase kinase kinase, raf-1, which phosphorylates and activates the MAP kinase kinases, MEK1/MEK2, the immediate upstream regulators of ERK1/ERK2 (20). To determine whether ERK1/ERK2 are involved in the actions of 1,25D, we first investigated whether 1,25D-stimulated the activities of ERK1/ERK2 in COS-1 cells. It is evident from the data in Fig. 4*A* that 1,25D at 10^{-7} M rapidly increased ERK1/ERK2 dual phosphorylation and hence activity. Although increased ERK1 phosphorylation persisted for at least 30 min, ERK2 phosphorylation peaked at 5 min after the addition of 1,25D. The effect of 1,25D on ERK1/ERK2 dual phosphorylation was also observed at 10^{-9} but not at 10^{-10} M (Fig. 4*B*). The precursor, 25D,

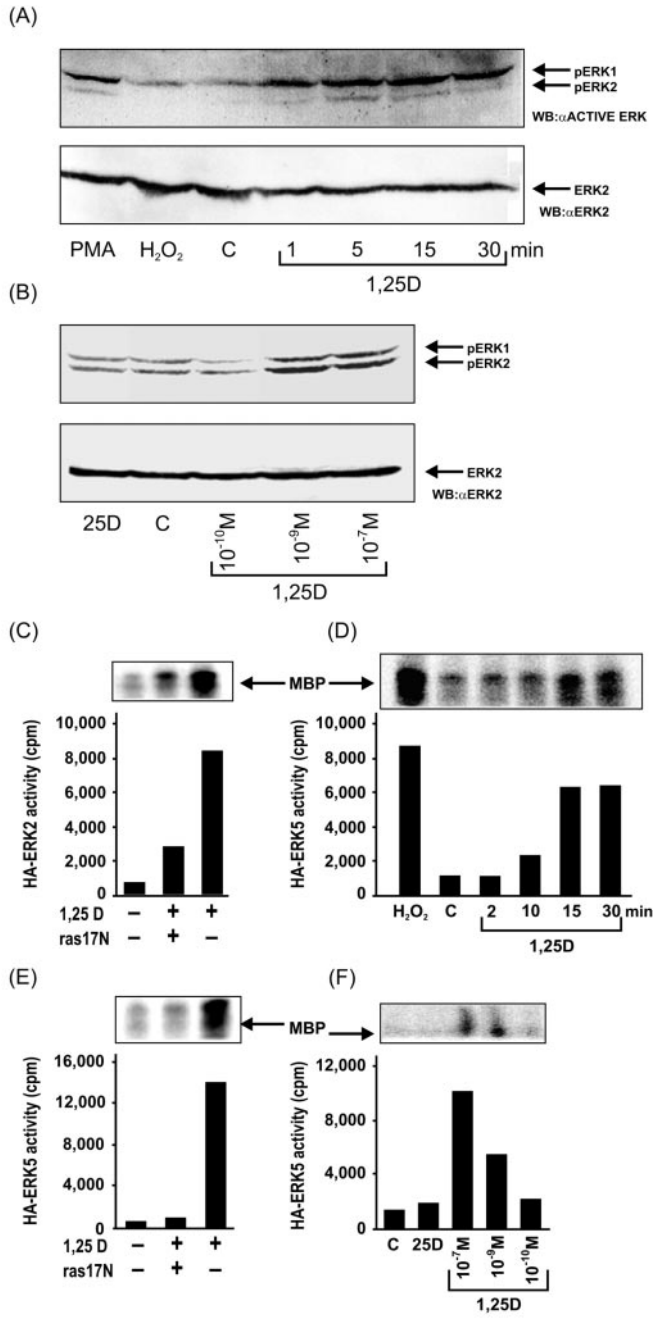

FIG. 4. **Activation of ERK1/ERK2 by 1,25D.** *A*, time-dependent stimulation of ERK1/ERK2 activities by 1,25D. COS-1 cells were stimulated with positive control phorbol 12-myristate 13-acetate (*PMA*, 10^{-7} M), negative control H_2O_2 (10^{-3} M), or 1,25D (10^{-7} M) for 5, 5, and 1–30 min, respectively. Ethanol solvent only is shown as *C*. The cells were lysed, and the dual phosphorylation of ERK1/ERK2 was determined by Western blot (*WB*) analysis using an anti-active ERK antibody (*WB:αACTIVE ERK*). Unphosphorylated ERK2 was detected using anti-ERK2 antibody (*WB:αERK2*). *B*, effect of 25D and 1,25D concentrations on ERK1/ERK2 activities. COS-1 cells were stimulated with 25D (10^{-7} M) or 1,25D (10^{-10}-10^{-7} M) for 5 min and then lysed, and the dual phosphorylation of ERK1/ERK2 was determined as described in *panel A*. *C*, effect of Ras17N on 1,25D stimulation of ERK2 activity. COS-1 cells were transfected with HA-ERK2 or cotransfected with this plasmid and *ras*17N. After stimulation with 1,25D (10^{-7} M) for 5 min, the cells were lysed, and HA-ERK2 was immunoprecipitated. Kinase activity was determined using MBP as a substrate, and the results were quantitated using an Instant Imager. *D*, time-dependent stimulation of HA-ERK5 activity. COS-1 cells were transfected with HA-ERK5 and stimulated with H_20_2 (15 min) or 1,25D (10^{-7} M) for 2–30 min. After lysis, the activity of immunoprecipitated HA-ERK5 was determined using MBP as a substrate. *E*, effect of Ras17N on stimulation of HA-ERK5. COS-1 cells were transfected with HA-ERK5 or cotransfected with this plasmid and *ras*17N. After stimulation (15 min), the cells were lysed, and immunoprecipitated HA-ERK5 activity was determined as described above. *F*, effect of 25D and 1,25D concentrations on HA-ERK5 activity. COS-1 cells were transfected with HA-ERK5 and stimulated with 25D (10^{-7} M) or 1,25D (10^{-10}-10^{-7} M) for 5 min, and kinase activity was determined as described above. Results are representative of three to five experiments.

at 10^{-7} M did not affect ERK1/ERK2 phosphorylation (Fig. 4*B*) nor induce pCYP24WT(−298)-Luc expression (data not shown) in which stimulation of MAP kinase was selective for 1,25D at a low concentration. Activation of ERK2 activity by 1,25D using myelin basic protein as a substrate was confirmed in cells that had been transfected with HA-ERK2 (Fig. 4*C*). Co-transfection of the cells with *ras*17N reduced but did not abolish 1,25D-stimulated ERK2 activation (Fig. 4*C*). These data demonstrate that 1,25D stimulates the Ras-ERK1/ERK2 cascade in COS-1 cells. Recently the activity of another MAP kinase, ERK5, was reported to be regulated by Ras (35). The upstream regulators in the ERK5 cascade consist of the MAP kinase kinase, MEK5, and the MAP kinase kinase kinases, MEKK3 and Cot (36–38). In a parallel manner, 1,25D also substantially stimulated HA-ERK5 activity after exposure of the cells to 1,25D for at least 10 min (Fig. 4*D*). Consistent with ERK5 being a stress-activated kinase (36), its activity was up-regulated by H_2O_2 (Fig. 4*D*). This is in contrast to the lack of effect of H_2O_2 on ERK1/ERK2 dual phosphorylation (Fig. 4*A*). The ability of 1,25D to stimulate ERK5 activity was essentially abrogated by Ras17N (Fig. 4*E*). As has been observed for ERK1/ERK2 dual phosphorylation, ERK5 activity was stimulated by 10^{-7} and 10^{-9} M 1,25D, whereas kinase activity was only marginally stimulated by 10^{-10} M (Fig. 4*F*). 25D at 10^{-7} M did not affect ERK5 activity (Fig. 4*F*). In these assays, basal ERK1/ERK2 and ERK5 phosphorylation and/or activities were detectable in unstimulated cells. In other experiments (data not shown), we observed that 1,25D at concentrations as low 10^{-10} M was able to activate pCYP24WT (298)-Luc, but 25D failed to activate even at 10^{-7} M.

Involvement of the ERK1/ERK2 and ERK5 Pathways—The above data raise the possibility that ERK1/ERK2 and ERK5 could be involved in the regulation of *CYP24* promoter induction by 1,25D. Preincubation of COS-1 cells with PD98059, an inhibitor of MEK1/MEK2 and MEK5 and thereby the respective ERK1/ERK2 and ERK5 modules (29), severely inhibited 1,25D induction of *CYP24* promoter activity when tested at 10 μM and almost abolished induction at 100 μM (Fig. 5*A*). The inhibitor U0126, which inhibits MEK1/MEK2 and MEK5, also markedly suppressed 1,25D induction, although not to the level observed for PD98059 (Fig. 5*A*). Furthermore, overexpression of the dual specificity MAP kinase phosphatase MKP-1 (31) was highly effective in blocking 1,25D induction (Fig. 5*A*). In contrast, neither SB203580 (10 μM), an inhibitor of p38α, -β, and -δ, nor the inhibitory dominant-negative mutant of MEKK1 (DN-MEKK1), a MAP kinase kinase kinase of the JNK cascade, affected 1,25D induction of the pCYP24WT(−298)-Luc construct (Fig. 5*A*). In these experiments, the chemical inhibitors and dominant-negative mutant at the concentrations used did not alter basal expression of pCYP24WT(−298)-Luc or pRL-TK-Luc (results not shown), demonstrating specificity in their inhibitory actions. From these data, we conclude that either the ERK1/ERK2 or ERK5 modules or both, but not p38 or JNK modules, mediate the 1,25D induction of the *CYP24* promoter in COS-1 cells. We investigated whether these requirements for 1,25D induction are unique to the *CYP24* promoter in these cells. The effects of the pharmacological inhibitors were determined on the expression of a plasmid

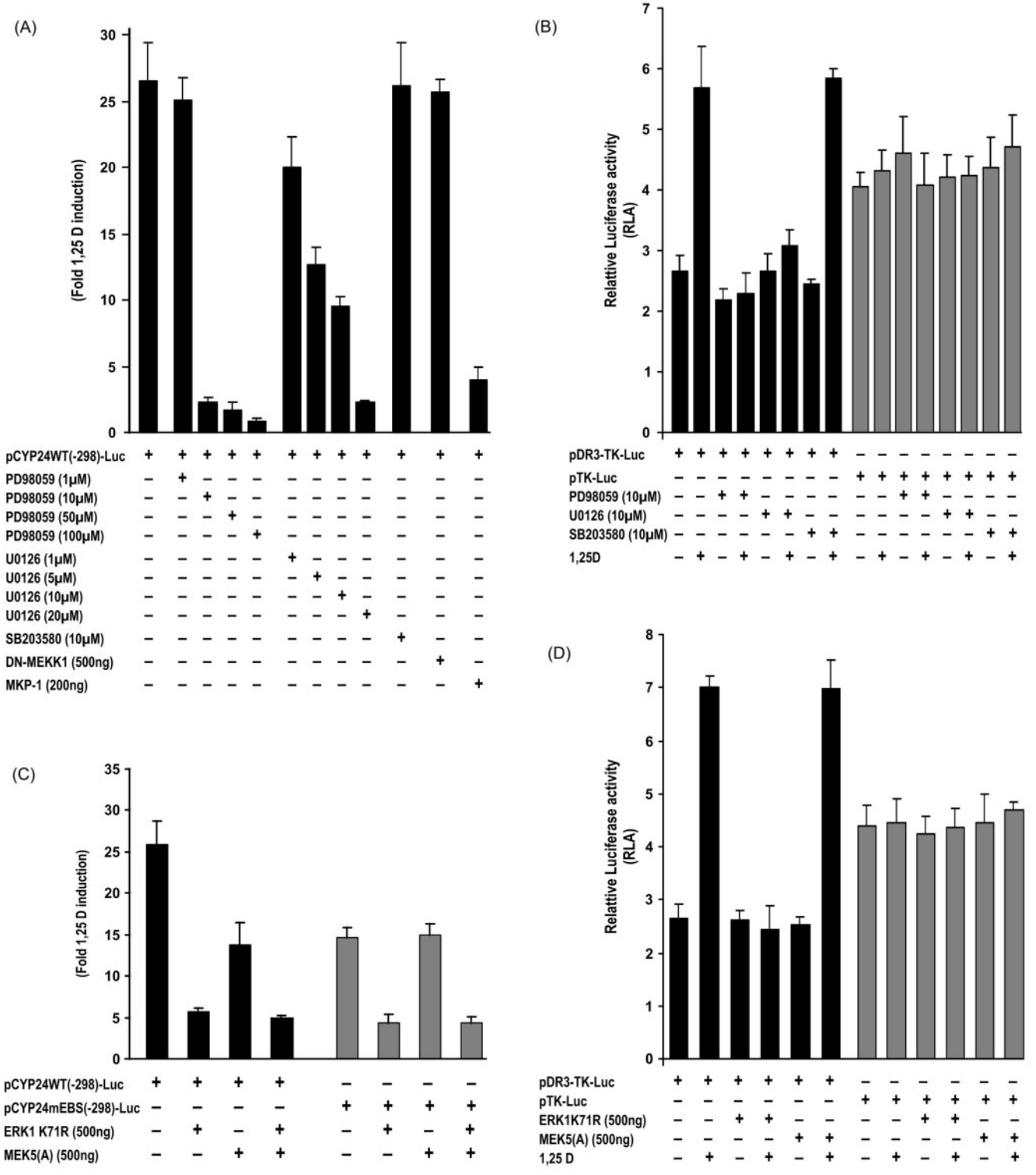

FIG. 5. **Involvement of ERK1/ERK2 and ERK5 pathways.** *A*, effect of specific MAP kinase pathway inhibitors, dominant negatives, and MAP kinase phosphatase on transactivation. The pCYP24WT(−298)-Luc construct was analyzed for its 1,25D-mediated transactivation, as in *panel A* of Fig. 2, in the presence of the MEK inhibitors (*PD98059* or *U0126*), the p38 inhibitor (*SB203580*), the dominant-negative MEKK1 (*DN-MEKK1*), or MKP-1. *B*, roles of specific MAP kinase pathways in the action of VDR. A consensus DR3-type VDRE sequence attached to the thymidine kinase promoter (*DR3TK-Luc*) was analyzed in the presence of the MEK inhibitors (*PD98059* or U0126), the p38 inhibitor (*SB203580*), as described in *panel A* above. The data were presented as relative luciferase activity (*RLA*). The data presented here are the averages of three independent experiments ± S.D. *C*, mediation of ERK1/ERK2 and ERK5 functions through EBS. The effect of 1,25D-mediated transactivation was analyzed, as in *panel A* of Fig. 2, on pCYP24WT(−298)-Luc and pCYP24mEBS(−298)-Luc constructs in the presence of the dominant-negative ERK1 (*ERK1 K71R*) and the dominant-negative MEK5 (*MEK5*(*A*)) constructs. *D*, roles of ERK1/ERK2 and ERK5 in the action of VDR. A consensus DR3-type VDRE sequence attached to thymidine-kinase promoter sequence (*DR3TK-Luc*) was analyzed in the presence of ERK1K71R or MEK5(A) as described above. The data were presented as relative luciferase activity (*RLA*). The data presented here are the averages of three independent experiments ± S.D. Fold 1,25D induction for *panels A* and *C* was calculated as in *panel A* of Fig. 2. The data presented here are the averages of three independent experiments ± S.D.

containing a DR3-type VDRE fused to the TK promoter (pDR3-TK-Luc) and the same plasmid lacking the VDRE (pTK-Luc) (Fig. 5*B*). 1,25D induced expression of pDR3-TK-Luc by about 2-fold, and this was inhibited by PD98059 and U0126 but not by SB203580. By contrast, these inhibitors did not affect expression of the pTK-Luc construct (Fig. 5*B*). In these experiments, lowered basal expression of pDR3-TK-Luc as compared with pTK-Luc was seen as observed previously and attributed to binding of a repressor to the VDRE in the absence of 1,25D (9). The data show that the requirement for ERK1/ERK2 and/or ERK5 pathways for 1,25D induction is not *CYP24* promoter-specific.

To further evaluate the contributions of the ERK1/ERK2 and ERK5 modules to 1,25D induction of the *CYP24* and TK promoter constructs, cells were transiently transfected with the dominant-negative mutant of ERK1 and MEK5, ERK1K71R and MEK5(A), respectively. ERK1K71R, which blocks the action of ERK1 and ERK2 (27), lowered 1,25D-induced *CYP24* promoter activity by about 80% (Fig. 5*C*). It is evident from these data and the results in Fig. 4 that the activity of the ERK1/ERK2 module is stimulated by 1,25D and also that the ERK1/ERK2 module constitutes a signaling pathway that is required for the induction of *CYP24* promoter activity by the hormone. MEK5(A), a specific inhibitory dominant-negative construct of the ERK5 pathway (29), reduced promoter activity by about 40% (Fig. 5*C*). The magnitude of this effect was similar to that caused by the EBS mutant promoter construct (Fig. 5*C*). The lowered 1,25D-inductive action observed for the EBS mutation was further reduced by ERK1K71R, but no further inhibition of the mutated EBS promoter construct was observed in the presence of the MEK5 inhibitory dominant-negative construct MEK5(A) (Fig. 5*C*). This latter finding is significant and establishes that ERK5 activity is specifically required for functionality of the EBS binding protein. In contrast, ERK1/ERK2 must be required for the phosphorylation and activation of other components of the induction machinery, which represent the principal signaling pathway in the induction process. In keeping with this concept, the MEK5 dominant-negative construct MEK5(A) did not further inhibit the 1,25D-inductive action in the presence of ERK1K71R (Fig. 5*C*). It is relevant that ERK1K71R, but not MEK5A, abolished 1,25D induction from the pDR3TK-Luc construct, which lacks the EBS (Fig. 5*D*). There was no effect of either ERK1K71R or MEK5A on expression of pTK-Luc, which also lacks both an EBS and a VDRE (Fig. 5*D*). Overall these results support the contention that the ERK1/ERK2 module is crucial in COS-1 cells for the 1,25D induction process, whereas ERK5 plays a more selective role in activating the EBS-stimulatory pathway.

Phosphorylation of RXRα by HA-ERK2—Possible ERK1/ERK2 substrates that are required for 1,25D induction of the *CYP24* promoter induction include RXRα, VDR, and coactivator complex components such as SRC-1. Consensus MAP kinase sites have been identified in SRC-1 (39) and RXR but not in VDR (40). In Ras-transformed keratinocytes, phosphorylation of RXRα at serine 260, which is within one of the two MAP kinase consensus sites, results in a non-functional VDR·RXR complex (40). However, the identity of the MAP kinase that phosphorylated this residue was not determined (40). We transfected COS-1 cells, therefore, with wild type RXRα or the mutant, RXRα S260A, in which the serine residue was replaced with alanine (Fig. 6*A*). Induction of the *CYP24* promoter by 1,25D was not influenced by overexpression of wild type RXRα (presumably reflecting high endogenous levels of RXR). In contrast, the mutant RXRS260A inhibited 1,25D-directed induction, which was an unexpected finding based on a previous study (40) in which the mutation prompted an enhanced 1,25D action. These results suggest that phosphorylation of RXRα at serine 260 is required for RXRα functionality during 1,25D induction of the *CYP24* promoter in COS-1 cells. When another potential MAP kinase site in RXR was mutated (threonine 82 to alanine), the expression of this mutant had no effect on 1,25D induction of the *CYP24* promoter construct (data not shown).

To investigate whether ERK1/ERK2 phosphorylated RXRα, COS-1 cells were transfected with HA-ERK2, and the cells were stimulated with 1,25D. The data in Fig. 6*B* demonstrate that HA-ERK2 immunoprecipitated from these cells phosphorylated wild type GST-RXRα. In contrast, parallel experiments using HA-ERK5-transfected cells showed that HA-ERK5 immunoprecipitated from 1,25D-stimulated cells failed to phosphorylate GST-RXRα (Fig. 6*B*). These results demonstrate substrate specificity for ERK2 and ERK5. The ability of activated HA-ERK2 to phosphorylate GST-RXRα was substantially reduced when serine 260 in RXRα was replaced with alanine (GST-RXRαS260A) (Fig. 6*C*). Hence, ERK2 activated by 1,25D can phosphorylate RXRα at serine 260. As expected, neither HA-ERK2 nor HA-ERK5 phosphorylated GST-VDR (Fig. 6*D*). Thus, VDR is not a substrate for either ERK2 or ERK5.

Interaction of RXRα with ERK2 but Not with ERK5 and Serine Phosphorylation of RXRα—Although the above observations demonstrate the selective action of ERK1/ERK2, it is not clear whether endogenous RXRα is phosphorylated in 1,25D-stimulated COS-1 cells and whether ERK1/ERK2 is involved in phosphorylating RXRα. To address this, COS-1 cells were transfected with HA-RXRα and stimulated with 1,25D. After lysis, the samples were subjected to immunoprecipitation with anti-HA antibody followed by Western blot analysis. The data in Fig. 7*A* demonstrate that ERK2 but not ERK5 co-immunoprecipitated with HA-RXRα, implying an interaction between ERK2 and RXRα. This interaction was not dependent on 1,25D (Fig. 7*A*). However, 1,25D increased the level of serine phosphorylation of RXRα that was prevented by the dominant-negative ERK1K71R and not observed with the mutant HA-RXRS260A construct (Fig. 7*B*).

Interaction of Ets-1 with ERK5 and Threonine Phosphorylation of Ets-1—Suppressed 1,25D induction of *CYP24* promoter activity by the inhibitory dominant-negative mutant MEK5(A) was not further inhibited by an EBS mutation, which implies that the MEK5-ERK5 module may target Ets-1 in a linear signaling pathway. To investigate this possibility, COS-1 cells were transfected with HA-Ets-1 and stimulated with 1,25D. After lysis, HA-Ets-1 was immunoprecipitated, and the samples were subjected to Western blot analysis. It is evident from the data in Fig. 8*A* that ERK5 interacted with Ets-1 in a 1,25D-dependent manner. Although ERK5 did not interact with Ets-1 in unstimulated cells, a weak interaction could be observed at 2 h, and strong interaction was clearly observed at 24 h (Fig. 8*A*, *upper panel*). A weak interaction between Ets-1 and ERK2 was also detectable, but this was not affected by 1,25D (Fig. 8*A*, *bottom panel*). Treatment of the cells with 1,25D caused a gradual increase in the level of threonine phosphorylation of Ets-1, detectable at 2 h and increasing over a 24-h incubation period (Fig. 8*B*, *bottom panel*). In addition, *in vivo* labeling experiments using $^{32}P_i$ revealed a 1.8-fold increase in Ets-1 phosphorylation in 1,25D-treated cells when examined after 4 h (data not shown).

Phosphorylation of Ets-1 by HA-ERK5—The possibility that the MEK5-ERK5 module may target Ets-1 in a linear signaling pathway and that Ets-1 is a substrate for ERK5 was investigated further using *in vitro* kinase assays. The HA-ERK5 that was immunoprecipitated from 1,25D-stimulated cells phosphorylated His-Ets-1 in a manner that was dependent upon Ras

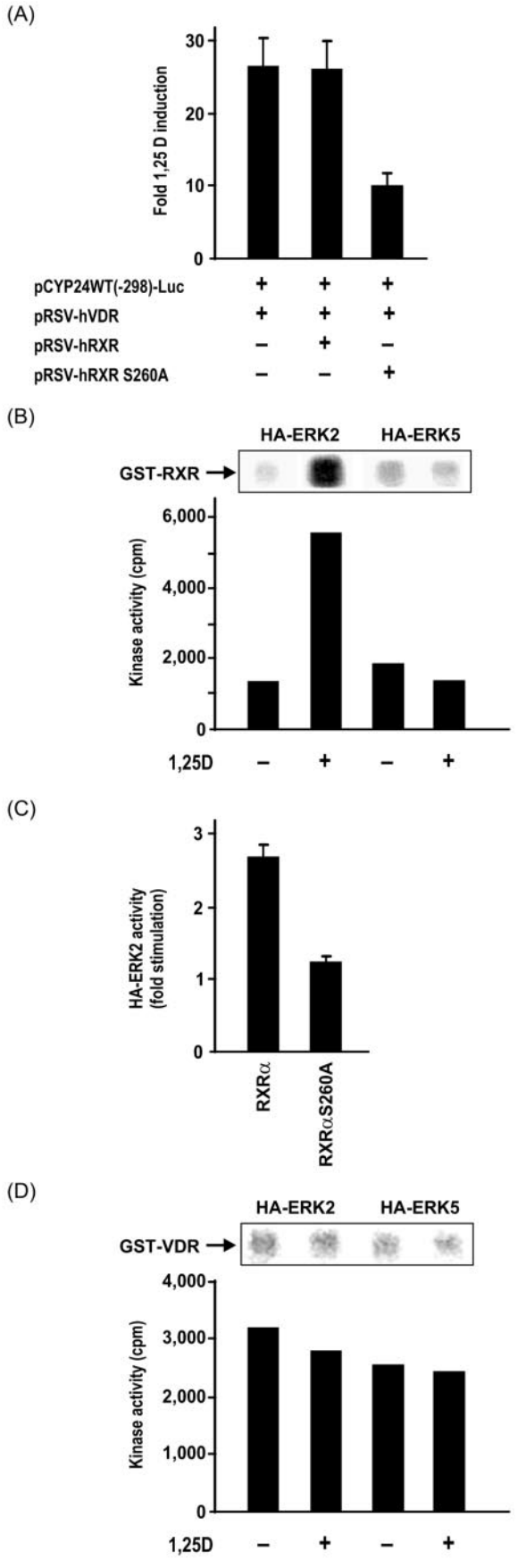

FIG. 6. **Phosphorylation of RXR*α* by HA-ERK2.** *A*, effect of pRSV-hRXR S260A on 1,25D induction of *CYP24* promoter. pCYP24WT(−298)-Luc, pRSVhVDR, pRSVhRXR, or pRSVhRXR S260A were cotransfected in COS-1 cells as indicated. Fold 1,25D induction was calculated as in *panel A* of Fig. 2. The data presented here are the averages of three independent experiments ± S.D. *B*, phosphorylation of GST-RXR by HA-ERK2 and HA-ERK5. For kinase activity assays, cells were transfected with HA-ERK2 or HA-ERK5 and stimulated with 1,25D as described in the legend for Fig. 4. The substrate was GST-hRXR. *C*, phosphorylation of GST-hRXR S260A by HA-ERK2. As in *panel B*, with GST-hRXR or GST-hRXR S260A as substrates. *D*, phosphorylation of GST-VDR by HA-ERK2 and HA-ERK5. As in *panel B*, with GST-VDR as substrate. All results are representative of three experiments.

and MEK5 (Fig. 9*A*). In contrast, HA-ERK2 immunoprecipitated from the lysate of 1,25D-stimulated cells failed to phosphorylate His-Ets-1 (Fig. 9*A*). The ability of Ets-1 to function as a phosphorylation target for ERK5 was greatly reduced by substituting threonine 38 for alanine in GST-Ets-1-T38A (Fig. 9*B*), suggesting that threonine 38 of Ets-1 is a specific target for ERK5.

DISCUSSION

Ets-1 plays a significant role in the up-regulation of *CYP24* promoter activity. The Ets binding site (EBS) in the rat *CYP24* promoter cooperates transcriptionally with VDRE-1 but not with VDRE-2, presumably reflecting the close proximity of the VDRE-1 and EBS (see Fig. 1). Indeed, direct *in vivo* protein-protein interaction between Ets-1 and VDR was demonstrated in the current study by the mammalian two-hybrid assay and recently by an *in vitro* GST-protein pull-down study (34). It is known that the proximal VDRE-1 is employed preferentially at low 1,25D concentrations, whereas both VDREs are utilized at high concentrations in a synergistic manner, providing the promoter with the flexibility to respond to both low and potentially toxic levels of hormone (16). Thus, the functional cooperation between the EBS and VDRE-1 that is established in the present study is likely to play an important role in facilitating efficient transcription of the *CYP24* gene across a broad range of 1,25D levels.

The signaling mechanisms that regulate the 1,25D-mediated transcriptional activation of the *CYP24* promoter are poorly defined. We previously suggested that Ras could play a role in regulating the induction of *CYP24* by 1,25D since overexpression of Ras enhanced hormone-stimulated *CYP24* promoter activity (18). The data presented here establish for the first time that Ras and the downstream MAP kinase pathways ERK1/ERK2 and ERK5 are critical for induction of the *CYP24* promoter in response to 1,25D in COS-1 cells. The involvement of Ras and ERK pathways was inferred from *CYP24* promoter expression studies using pharmacological antagonists and specific inhibitory dominant-negative mutants coupled with the demonstration that 1,25D stimulated the activities of ERK1/ERK2 and ERK5 in a Ras-dependent manner. ERK5 was observed to act specifically through the EBS. However, ERK1/ERK2 was observed to function at one or more locations other than the EBS since the ERK1 dominant-negative mutant ERK1K71R severely inhibited induction of the promoter construct beyond the inactivation observed for a mutated EBS. Furthermore the observation that ERK1K71R, but not the MEK5 inhibitor MEK5(A), abolished induction of the artificial TK-VDRE promoter construct (*i.e.* no EBS) strongly implicates an ERK1/ERK2-dependent phosphorylation step that involves the VDRE and is vital in the 1,25D induction mechanism. Neither the JNK nor the p38 modules contributed to *CYP24* induction in these cells.

The identification of Ets-1 as a substrate of ERK5 adds to the list of transcription factors that are substrates of ERK5. Previous studies have demonstrated that ERK5 phosphorylates c-Myc, Sap1a, and members of the MEF2 family of transcription factors such as MEF2C (29, 36, 41). ERK5 has also been

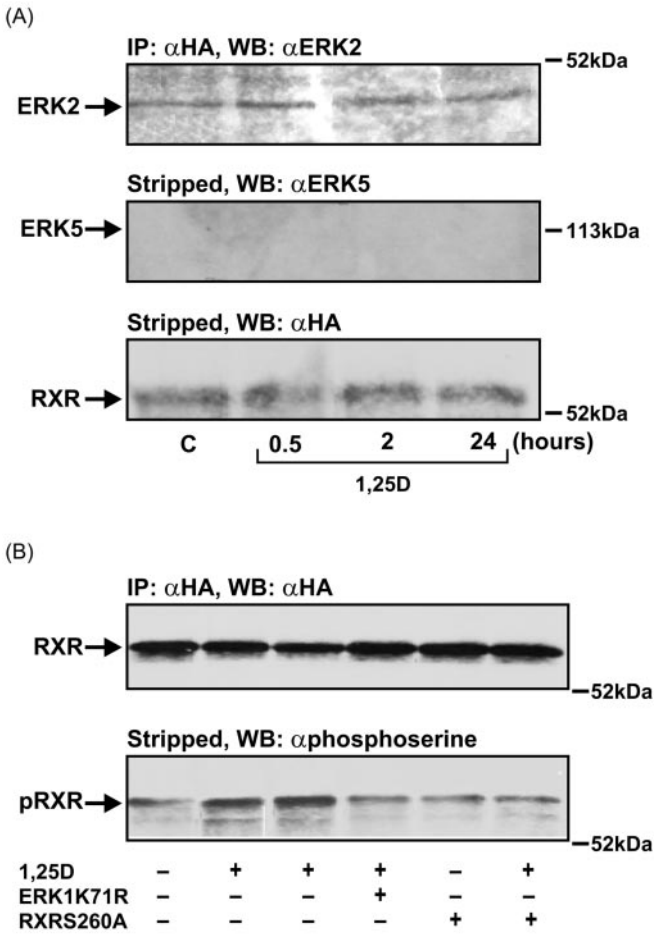

FIG. 7. **Interaction of RXRα with ERK2 and serine phosphorylation of RXRα.** *A*, interaction of RXR with ERK2 in intact cells. COS-1 cells were transfected with HA-RXRα and incubated with 1,25D (10^{-7} M) for 0.5–24 h or solvent only (*C*). After lysis, HA-RXRα was immunoprecipitated with an anti-HA antibody and Western blotted (*WB*) to detect the presence of co-immunoprecipitated ERK2. Blots were stripped to enable reprobing with ERK5 antibody. The presence of RXRα was confirmed by probing the blots with an anti-HA antibody. *B*, anti-phosphoserine antibody detection of phosphorylated RXR. COS-1 cells were cotransfected with HA-RXRα and ERK1K71R or HA-RXRS260A before being stimulated with 1,25D (2 h), and after lysis, cells were immunoprecipitated with an anti-HA antibody and Western blotted for the presence of RXRα, and then blots were stripped and reprobed with an anti-phosphoserine antibody to determine the level of RXRα phosphorylation. Results are representative of two (*panel A*) or three (*panel B*) experiments.

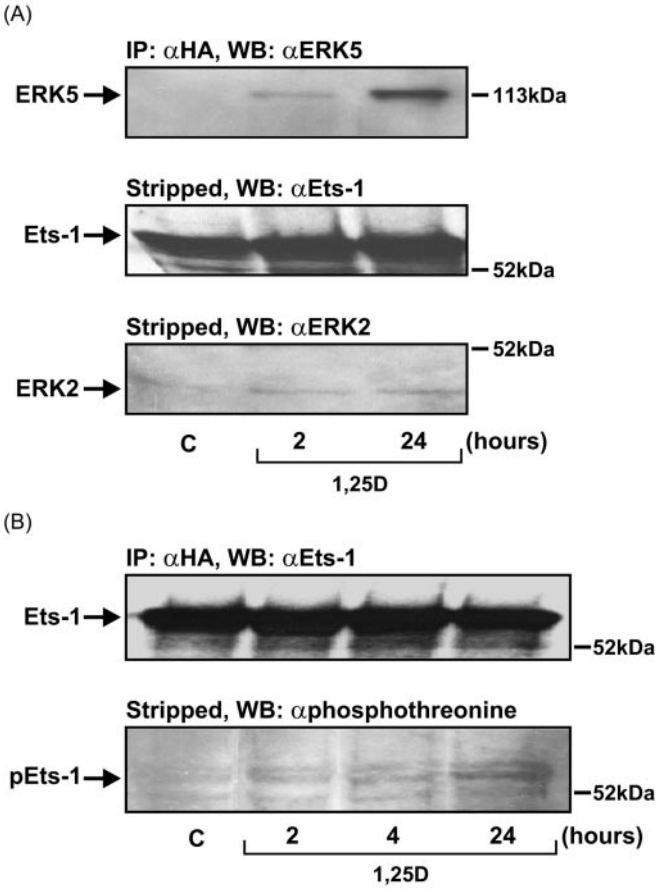

FIG. 8. **Interaction of Ets-1 with ERK5 and threonine phosphorylation of Ets-1.** *A*, 1,25D-dependent interaction of ERK5 with Ets-1. COS-1 cells were transfected with HA-Ets-1 and incubated with 1,25D (10^{-7} M) for 2–24 h or with solvent only (*C*). After lysis, HA-Ets-1 was immunoprecipitated with an anti-HA antibody, and the samples were subjected to Western blot (*WB*) analysis to detect the presence of co-immunoprecipitated ERK5, Ets-1, and ERK2. Blots were stripped to enable reprobing with another antibody. *B*, threonine phosphorylation of Ets-1 in intact cells. As in *panel A*, except samples were blotted and probed for the presence of Ets-1 using anti-Ets-1 antibody, stripped, and probed with an anti-phosphothreonine antibody to determine the level of Ets-1 phosphorylation (*middle band*). Results are representative of three experiments.

reported to be involved in regulating the expression of tumor necrosis factor α in mast cells, although the mechanisms through which ERK5 achieved this were not investigated (42). Ets-1 contains a consensus MAP kinase phosphorylation site at threonine 38 (33), and mutagenesis of the threonine residue to alanine severely abrogated the transcriptional cooperation between EBS and VDRE-1 in response to 1,25D (18). In view of the importance of threonine 38 in Ets-1 function, it is significant that mutagenesis of threonine 38 also abolished the ability of activated HA-ERK5 to phosphorylate this substrate. This observation establishes that ERK5 phosphorylates Ets-1 on threonine 38, and the phosphorylation of this residue has a functional role in facilitating the transcriptional cooperation between EBS and VDRE-1. Our finding that expression of the MEK5 dominant-negative MEK5(A) inhibited ERK5 activation and *CYP24* promoter activity is consistent with the concept that the ERK5 module couples the action of 1,25D-liganded VDR to the EBS via Ets-1. This is reinforced by our demonstration in intact cells that 1,25D not only caused an increase in the threonine phosphorylation of Ets-1 but that it also promoted the binding of ERK5 to Ets-1. In contrast, 1,25D did not have any impact on the weak but detectable interaction between ERK2 and Ets-1. An interaction between ERK2 and Ets-1 is perhaps not surprising because Ets-1 has an LXL ERK2 docking site C-terminal to the threonine 38 MAP kinase phosphoacceptor. In contrast to the recent demonstration that Ets-1 is a substrate for bacterially expressed ERK2 in cell-free assays (43), it is apparent from our data that ERK2 immunoprecipitated from 1,25D-stimulated cells did not phosphorylate Ets-1. Suggested from this apparent discrepancy is the possibility that ERK2 expressed and activated in mammalian cells (our study) behaves differently from recombinant enzyme that is expressed and activated in bacteria (43).

Our demonstration that ERK5 did not interact with RXRα in intact cells could account for the inability of 1,25D-activated HA-ERK5 to phosphorylate RXRα in *in vitro* kinase assays despite the presence of two MAP kinase phosphoacceptor sites (*i.e.* threonine 82 and serine 260 on RXRα) (40). It would appear, therefore, that RXRα could lack an appropriate ERK5 docking site. On the other hand, 1,25D clearly activated the HA-ERK2 phosphorylation of RXRα. Investigations in intact

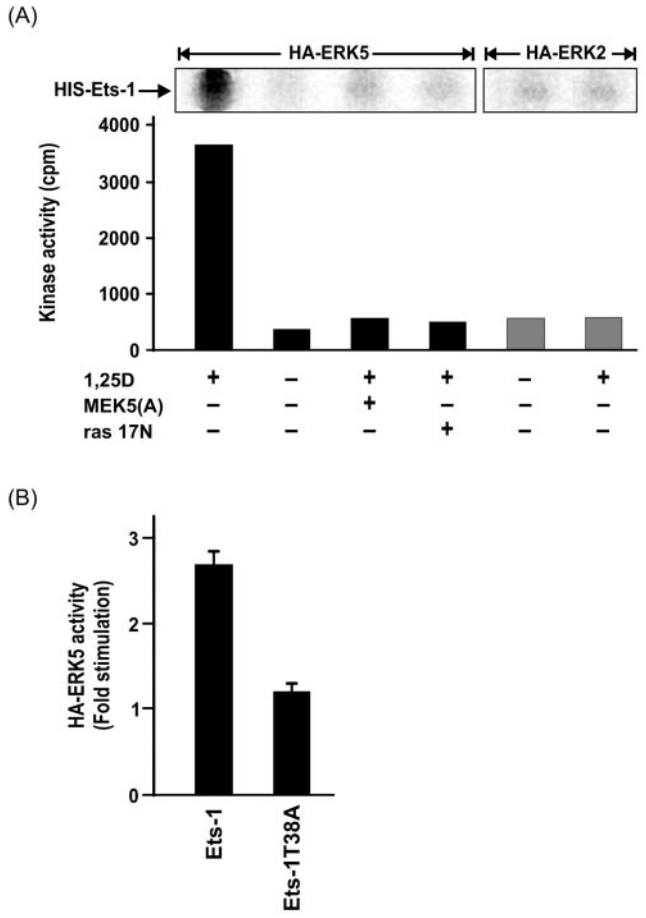

FIG. 9. **Phosphorylation of Ets-1 by HA-ERK5.** *A*, phosphorylation of HIS-Ets-1 by HA-ERK5 in a Ras- and ERK5-dependent manner. COS-1 cells transfected with either HA-ERK2 or HA-ERK5 were stimulated with 1,25D for 5 min (*HA-ERK2*) or 15 min (*HA-ERK5*). Some cells were also co-transfected with either *ras*17N or MEK5(A). After lysis, HA-tagged kinase was immunoprecipitated, and kinase activity was determined using HIS-Ets-1. *B*, phosphorylation of wild type and mutant Ets-1, as in *panel A* but using GST-Ets-1 or GST-Ets-1 T38A as a substrate. Results are representative of three experiments.

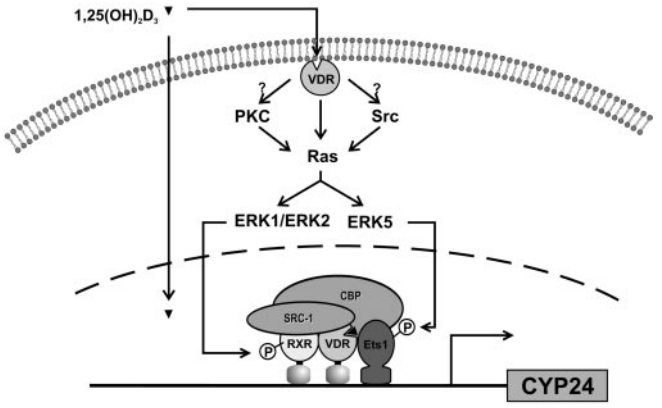

FIG. 10. **Proposed model for transcriptional regulation of *CYP24* promoter by 1,25D-mediated nongenomic and genomic actions in COS-1 cells.** We hypothesize that nuclear VDR is also located at the plasma membrane. The mechanism by which liganded VDR at the membrane activates ERK1/ERK2 and ERK5 remains to be established, although Ras is involved possibly through protein kinase C (*PKC*) or Src kinase activities. Phosphorylated residues in RXRα and Ets-1 could interact with the coactivators SRC-1 and CREB-binding protein (CBP), which themselves could be targets of ERK activity.

cells revealed that ERK2 interacted with RXRα, although this interaction was not regulated by 1,25D. This specificity of the MAP kinases for Ets-1 and RXRα is in direct contrast to their lack of specificity toward certain other substrates. For example, promiscuous substrates such as myelin basic protein and Sap1a are phosphorylated by ERK1/ERK2, ERK5, and p38 (29, 44). The specificity of a MAP kinase for its target sites can also be observed between two phosphoacceptor sites within the same molecule. For example, threonine 82 and serine 260 within RXRα are both consensus MAP kinase phosphorylation sites (40), yet mutating serine 260 to alanine nearly totally abolished the ability of activated HA-ERK2 to phosphorylate RXRα. This implies that serine 260 in RXRα is the preferred phosphorylation site of ERK1/ERK2. Other examples of specificity include the recent demonstration that the ERK5-related kinase p97, but not ERK5, phosphorylates Ets-2 (45), although Ets-2 is also a substrate for ERK2 (43). Docking domains that are known to enhance the efficiency of substrate phosphorylation are likely to play significant roles in determining specificity (46). Analysis of the MAP kinase docking domains on RXRα may provide clues to the reasons for ERK5 not being able to interact with and phosphorylate RXRα as well as why serine 260 is the preferred phosphorylation site for ERK2.

The selective targeting of RXRα and Ets-1 by ERK1/ERK2 and ERK5, respectively, implies that the ERKs act via distinct *cis* elements and coregulatory complexes on the *CYP24* promoter. Consistent with this observation, expression of the dominant-negative MEK5(A) mutant, mutagenesis of threonine 38 to alanine, or the loss of EBS function attenuated *CYP24* promoter activity by similar levels. Expression of the serine 260 to the alanine RXRα mutant inhibited 1,25D induction of the *CYP24* promoter, although the magnitude was not as great as that observed with the expression of the ERK1 dominant-negative mutant, ERK1K71R, implicating the involvement of other phosphorylated regulatory proteins. The possibility that low level phosphorylation of threonine 82 on RXRα by ERK1/ERK2 permitted some induction of the *CYP24* promoter activity was excluded on the basis that mutation of threonine 82 to alanine did not affect 1,25D-stimulated promoter activity.[2]

It is evident from the current study that serine 260 on RXRα is a functionally important ERK1/ERK2 phosphorylation site that facilitates 1,25D induction of the *CYP24* promoter in COS-1 cells. However, this observation is in direct contrast to Ras-transformed keratinocytes in which phosphorylation of serine 260 attenuated transactivation by 1,25D and rendered the cells insensitive to 1,25D-induced apoptosis (40). Moreover, the 1,25D induction of a transfected VDRE-containing reporter gene construct in keratinocytes was stimulated by the MAP kinase inhibitor PD98059 (40). This finding is in marked contrast to the inhibitory effect of PD98059 on 1,25D-dependent induction of the native *CYP24* promoter and the artificial VDRE-driven TK promoter in COS-1 cells. The molecular basis for this difference in the action of phosphorylated RXRα in the different cell types is not known. Nevertheless, the data imply that the role(s) played by ERK1/ERK2-phosphorylated RXRα in the actions of 1,25D differs from cell type to cell type. However, it should be noted that the kinase(s) that phosphorylated serine 260 in RXRα in the keratinocyte study was not identified (40).

Although the mechanisms by which 1,25D activates the Ras-MAP kinase pathways are not known, the current studies establish a critical role for the Ras-ERK1/ERK2-RXRα signaling pathway in the induction of the *CYP24* promoter by 1,25D. The Ras-ERK5-Ets-1 pathway plays an enhancing role in promoter activation. Together, these MAP kinases provide a novel

[2] P. P. Dwivedi, C. S. T. Hii, A. Ferrante, J. Tan, C. J. Der, J. L. Omdahl, H. A. Morris, and B. K. May, unpublished data.

mechanism for the induction of *CYP24* by facilitating a functional cooperation between the Ets-1 binding site and the proximal vitamin D response element in the presence of 1,25D. The data in the present study (summarized in Fig. 10) not only establish a novel role for ERK5 but also provide a stepping stone for investigating the mechanisms of action for 1,25D in other hormone-responsive cell types.

The rapid response of 1,25D to activate ERK1/ERK2 and ERK5 is consistent with nongenomic functions that have been attributed to several actions of the hormone (22, 23). A key question in the current study, however, concerns the underlying molecular mechanism by which vitamin D activates MAP kinase pathways. It can be envisaged that a receptor is located at the cytoplasmic membrane and is responsible for activation of signaling molecules (23, 49) as revealed recently for the ERα estrogen receptor and the IGF-1 signal cascade (50). In this regard, we have recently shown that overexpression of pRSV-hVDR in COS-1 cells increases vitamin D induction of the *CYP24* promoter and activation of ERK1/ERK2.[2] This finding makes it attractive to suggest that the same isoform of VDR is present at nuclear and extranuclear locations, as noted in the schematic for regulation of the *CYP24* promoter by 1,25D (Fig. 10). The proposed regulatory scheme also accounts for the documented regulatory actions of 1,25D in other cellular systems in which the hormone functions through the protein kinase C or Src pathways (51, 52). Although the nature of the cytosolic 1,25D initiation event remains to be determined, it is apparent that the action of the hormone to regulate *CYP24* promoter activity in COS-1 cells is mediated predominately through a Ras-dependent pathway that involves activation of ERK1/ERK2 and ERK5 and possibly other undocumented downstream kinase effectors. Therefore, it is evident from the current study that the genomic action of 1,25D to control *CYP24* promoter activity is linked tightly to the MAP kinase pathways and phosphorylation-mediated activation of RXRα and Ets-1, whose regulatory functions are dependent upon associated coregulatory proteins (47, 48) (Fig. 10).

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